secreted proteins (9). We have shown that P. pastoris can also be effective for intracellular expression of a PTK. Our experiments define the appropriate combination of salt, pH and detergent required to prevent P. pastoris protein precipitation without compromising subsequent affinity purification steps.

Commonly, hosts such as E. coli or insect cells have been used for recombinant expression of PTKs (5,7,8,10). We found, however, that the c-kit catalytic domain was primarily insoluble when expressed in bacteria (unpublished), complicating purification and subsequent analysis. Although SF9 insect cells, in conjunction with baculovirus-based vectors, have been very useful as hosts for PTK expression, the existence of endogenous tyrosine kinase and phosphatase activity potentially limits the usefulness of these cells for this application. In conclusion, our results show that P. pastoris is a very convenient and useful host for expression of recombinant intracellular proteins, particularly PTKs.

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Generation of a Moveable Poly(A)+ Cassette

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It has become clear that sequences in the untranslated region of mRNAs exert profound effects on the stability, translatability and subcellular localization of transcripts (1,3). In particular, significant attention has been focused on the poly(A)+ tail. In vivo, the length of the poly(A)+ tail is often in flux and can influence the stability of mRNAs (4). During cDNA library construction, the poly(A)+ tail is often truncated or even lost, potentially altering the behavior of subsequently generated transcripts. For direct studies of the role of the poly(A)+ tail, it is desirable to have a defined number of adenine residues on each transcript under investigation to remove potential artifacts.

We report a method for generating cassettes of poly(A) tails that can be readily transferred between vectors for the generation of in vitro-transcribed mRNAs with poly(A) tails of defined lengths. This vector differs from other poly(A) vectors [such as the pSP64 plasmids (2)] in two respects: (i) the A/T tracts are generated by polymerization rather than resulting from reverse transcription cDNA cloning, and (ii) the cassette can be moved into other vectors already containing the gene of interest.

A modified pBluescript® KS vector (Stratagene, La Jolla, CA, USA) was used that contains an altered polylinker with the following restriction sites: BamHI/BglII/BamHI. (Vectors with inverted polylinker sites, such as M13mp7/pUC7, can also be used.) The pBluescript was linearized with BglII (all restriction enzymes from Promega, Madison, WI, USA) (Figure 1), and the ends were filled in using Klenow DNA Polymerase (Promega) according to the manufacturer’s recommendations. The DNA was precipitated and resuspended at 1 µg/µL in water. The linear, blunt-ended vector (2 µg) was incubated with 500 µM dATP and terminal transferase (30 U; Promega) in a final volume of 15 µL using the buffer provided. After a 60-min incubation at 37°C, the DNA was precipitated and the washed pellet resuspended in 10 µL ddH2O. To determine if the reaction was successful, an aliquot of the diluted DNA was digested with PvuII and size-fractionated in a 1.5% agarose gel. The addition of poly(A) tails resulted in a smear larger than the approximately 400-bp size of the untailed fragment. The tailing reaction was repeated using a second aliquot of the linear, blunt-ended pBluescript vector with 125 µm dTTP.

The two tailed vectors were then digested with ScaI, which cleaves within the ampicillin-resistance gene and produces two fragments of approximately 1 and 2 kb. After fractionation in a 1% Low Melting Point Agarose Gel (Life Technologies, Gaithersburg, MD, USA), the larger fragment with the adenine residues and the smaller fragment...
with the thymine residues were isolated and ligated together in a 10-µL reaction mixture (overnight at room temperature) using equal amounts of each fragment. The ligation was terminated by heating to 65°C, and the products were introduced into DH5α™ E. coli cells.

Cells were grown under ampicillin selection and white colonies selected for miniprep isolation. Plasmid DNA was digested with *Pvu*II and analyzed on a 1.5% agarose gel. Those recombinants with inserts greater than the anticipated 400 bp were selected for DNA sequencing using *T*3 and *T*7 primers and Sequenase® Version 1.0 (Life Technologies). Initial analysis of a subset of the recombinants revealed that one clone contained a poly(A) cassette containing approximately 60 adenine residues. The poly(A) cassette was liberated from the vector by digestion with *Bam*HI; however, the small size of this fragment (<70 bp) precluded visualization even in 2% agarose. Therefore, we isolated all the DNA in the lane from below 100 bp to the running front (a ladder was used to monitor size and migration). The fragment was then precipitated and introduced into a *Bam*HI site 3′ to the gene of interest.

Using this cassette, we were able to study the effects of the poly(A) tail on the half-life and translatability of a death-associated message (unpublished). This method could also be used to generate polynucleotide tracts that could be relocated within a DNA to study the effects of sequence-induced structural changes in nucleic acids.

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Detection and Analysis of Living, Growth-Inhibited Mammalian Cells Following Transfection

At present, it is possible to demonstrate the effects of growth-inhibitory genes by co-transfection of such genes along with resistance genes into mammalian cells followed by drug selection (3). It usually takes at least two weeks of selection until resistant colonies can be identified and quantitated. It is also possible to evaluate the effects of growth-inhibitory genes in transient transfections. Immunohistochemical or fluorescence techniques that identify the protein product of the transfected gene and co-transfection of a second